JOURNAL OF Applied Polymer SCIENCE

Preparation and Evaluation of Aconitine Imprinted Microspheres and Its Application to Body Fluid Samples

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ABSTRACT: To extract aconitine from body fluid samples, aconitine-molecularly imprinted polymer microspheres with the optimum molar ratios of template/monomer/cross-linker (1:8:40) as selective sorbents were synthesized by precipitation polymerization. Excellent retention of aconitine on the molecularly imprinted microspheres (MIMs) cartridge was achieved by optimizing the MISPE process, and the binding capacity reached 0.802mg/g, yielding an imprinting factor of 4.76. The MIMs also showed high selectivity for aconitum alkaloids, but not for other kinds of poisonous alkaloids. High recoveries (>89%) for aconitine, hypaconitine, and mesaconitine were got in spiked serum samples. The working curves show linear dependence on aconitine concentration in the range of 2.0–0.1 μg/mL, and the detection limits of aconitine, hypaconitine, and mesaconitine were 16.7, 18.3, 10.2 ng mL^{-1}, respectively.
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KEYWORDS: molecular imprinting; separation techniques; adsorption; applications

Received 5 April 2012; accepted 11 August 2012; published online
DOI: 10.1002/app.38462

INTRODUCTION

Aconitine, one of aconitum alkaloids from some Chinese medicinal herbs of genus aconitum in family of ranunculaceae, such as Chuanwu, Caowu, and Fuzi, is both intoxicant and active. These medical herbs were used to treat rheumatosis, rheumatoid arthritis, and some other inflammations. However, the strong toxicity of aconitine often causes poisoning incidents. Therefore, the development of a viable method for qualitative and quantitative analysis of aconitine in body fluids drawn from patients or victims is of particular significance.

Kinds of methods have been developed for the determination of aconitum alkaloids in body fluid samples in previous papers. Such as high-performance liquid chromatography (HPLC),[1,2] gas chromatography–mass spectrometry (GC/MS),[3] liquid chromatography-mass spectrometry (LC/MS),[4] LC/MS/MS,[5,6] and so on. Recently, a HPLC or LC/MS method combined with solid-phase extraction (SPE) has been developed for determination of the content of aconitum alkaloids in blood and urine samples.[1,2,7–9] The main problem associated with SPE columns packed with ordinary stationary phases (such as C_{18} or ion-exchange materials) is the low selectivity of the retention mechanism.[10] A desired grade of selectivity may be obtained by using columns packed with materials based on well-defined molecular recognition mechanisms, which are able to bind analytes with high selectivity.[11] Molecularly imprinted polymers (MIPs) appear to be well-suited for this application.

Molecularly imprinted polymer is an artificially synthesized macromolecular material, which has prearrangement of structure and specific molecular recognition ability.[12] The production of MIPs involves the synthesis of highly crosslinked polymers in the presence of template molecules. After removal of the template, the resultant cavities, which complement the template in size, shape, and arrangement of functional groups (from the functional monomer), are allowed to rebind to the template molecules.[13,14] There are several methods to prepare MIPs, including bulk polymerization,[15–17] precipitation polymerization,[18–21] suspension polymerization,[22] surface imprinting on silica spheres,[23–25] etc. Nevertheless, precipitation polymerization is widely accepted for its simplicity and easy implement. It can provide uniform and porous polymers with small particle diameters and large specific surface areas, and possess rapid mass transfer characteristics. Recently, the MIPs were commonly used as sorbents of molecularly imprinted solid-phase extraction (MISPE) to separate compounds from complex matrices, such as food,[26,27] plants,[28–30] environmental samples,[31–33] and body fluid samples.[34,35] Ricardo et al.[36] developed a method constituted by MISPE with high-performance liquid chromatography for cotinine analysis in saliva samples. Wu et al.[37] prepared MIMs based on a biologically inspired hydrogen-bond array, using allobarbital as the novel functional monomer and simetryne as the template. Furthermore, they used the MIMs as a sorbent for selective extraction of simetryne from corn and soil samples by molecularly imprinted solid phase extraction.

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In this article, aconitine molecularly imprinted microspheres (MIMs) were synthesized for the first time. The MIMs were evaluated and then applied to separate aconitum alkaloids in human serum and urine samples selectively. The eluate of MISPE was quantified by HPLC finally.

EXPERIMENT

Reagents

Aconitine, mesaconitine, and hypaconitine (purity > 98%) were provided by Sinuote Biological Technique Company (Shanxi, China). Vauqueline and camptothecin (purity > 98%) were purchased from Guanyu Biological Technique Company (Xi'an, China). Methacrylic acid (MAA, chemically pure) was obtained from Guangzhou Xingang Reagent Factory (Gongdong, China). Ethylene glycol dimethacrylate (EGDMA) was purchased from Shanghai Shanhu Chemical Plant (Shanghai, China) and was distilled before being used to remove any stabilizers. 2, 2′-azobisisobutyronitrile (AIBN) was purchased from Shanghai Sihei Chemical Reagent Company (purity>99%, Shanghai, China). Methyl alcohol and acetic acid were purchased from Kermel Chemical Reagents Development Center (Tianjin, China). Acetonitrile and toluene were purchased from Guangzhou Chemical Reagent Factory (Guangdong, China). Camptothecin and vauqueline (purity > 98%) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). All reagents were of analytical grade or better.

Apparatus

The HPLC system was consisted of a Shimadzu LC-10AD pump (Kyoto, Japan) and a Shimadzu SPD-10A UV-Vis detector. All determinations were performed on an analytical, reverse-phased Symmetry C18 (4.6 mm I.D. × 15 cm long, 5 μm) column (Dikema) at a mobile phase flow rate of 0.5 mL min^{-1}, under isocratic conditions at room temperature. The mobile phase used for the HPLC experiments was a mixture of methanol : water : ammonium acetate : acetic acid (545:450:1.2:5.0, v/v/v/v), and was passed through a 0.45-μm filter prior to being used. The wavelength of the detector used for analyses was 240 nm. Supelco (USA) provided the SPE installation. The scanning electron microscope (SEM) micrograph was generated on a Hitachi S-520 (Kyoto, Japan). Fourier transform infrared (FTIR) spectra were recorded with IRPrestige-21 using a Spectrum One FTIR spectrometer (PerkinElmer Company, USA).

Preparation of MIMs

Totally, 0.2 mmol MAA, 1 mmol EDGMA and different amount of aconitine was used to optimize the molar ratios of template/monomer/cross-linker. Different porogens and different amounts of the porogens (5, 10, 15, 20, and 25 mL): toluene, acetonitrile, methanol, methylbenzene : acetonitrile (1:4 v/v), methylbenzene : acetonitrile (1:1 v/v), methylbenzene : acetonitrile (4:1 v/v), methylbenzene : methanol (1:4 v/v), methylbenzene : methanol (1:1 v/v), methylbenzene : methanol (4:1 v/v) were investigated to obtain the optimum porogen.

Then the MIMs were prepared by dissolving 0.2 mmol of template aconitine (0.129 g), 1.6 mmol of functional monomer MAA (0.138 g), and 8 mmol of crosslinker EGDMA (1.584 g) in 40 mL of toluene. After the mixture had been sonicated for 10 min, 0.080 g of initiator AIBN was added. Subsequently, the solution was transferred into three 20 mL glass tubes equally. The tubes were degassed with oxygen-free nitrogen for 15 min before sealed and thermostated at 30°C in a water bath for prepolymerization for 4 h. And then elevate the temperature to 60°C for polymerization for 48 h. The MIMs were under vacuum dried at 60°C for 8 h. Thereafter, the template and unreacted monomers were removed by Soxhlet extraction with a methanol-acetic acid (7:3, v/v) mixture until no template was detected in the washing solution. Non-imprinted microspheres (NIMs) were prepared in the same manner as the MIMs, but in the absence of the template.

MISPE Evaluation

Totally, 100 mg of dry MIMs or NIMs were suspended in methanol and packed into a 3 mL empty polypropylene SPE cartridge between two frits, in order to secure the packing and outlet stopcocks.

Before extraction, the cartridges were activated with 3 mL of methanol : acetic acid (7:3, v/v), followed by 5 mL of methanol to eliminate residual acetic acid and 3 mL of loading solvent to equilibrate the cartridges. Then, the analyte solution was loaded, washed and eluted. The eluate was collected and analyzed by HPLC. The measurements were performed three times.

Serum Samples

Spiked Serum Sample Preparation. Transfusion serum was used as the serum samples. Serum samples were to be taken precipitation protein with methanol (methanol : serum (5:5, v/v)). Totally, 1 mL methanol was added into a commensurate volume of serum sample which was spiked with certain concentration of analytes, and 0.02 mL of acetic acid was added. Then, the sample was mixed ultrasonically for 20 min, and then centrifuged in 1500r/min for 20 min. The supernatant was filtrated through a 0.45-μm filter and filtrate was obtained as loading solution for MISPE.

MISPE of Serum Samples. The MISPE cartridge was conditioned with 3 × 1 mL of methanol : water (5:5, v/v), and then 1 mL of prepared serum sample was loaded into the MISPE cartridge. Subsequently, the cartridge was washed with 3 × 1 mL of methanol : water (7:3, v/v) and 1 mL deionized water. The analytes were eluted from the MISPE cartridge with 2 × 1 mL of methanol : acetic acid (7:3, v/v). The eluate was collected and completely evaporated under vacuum. The residue was redissolved in 100 μL of methanol prior to analysis with HPLC. Experiments were performed in triplicate. To contrast with a C_{18}-SPE, a C_{18}-SPE procedure with the same process was handled.

Urine Samples Preparation

Urine samples were obtained from a normal volunteer. The spiked sample was used as loading solution in MISPE after being filtrated with 0.45-μm filter membrane. After conditioning the MISPE cartridge with 3 × 1 mL of methanol : water (5:5, v/v), the cartridge was loaded with 1mL of the prepared urine sample and washed with methanol : acetone : water (70:3:27, v/v/v). Likewise, after the eluting step, the eluent was collected, evaporated under vacuum and redissolved in 0.1 mL

methanol for HPLC analysis. Experiments were performed in triplicate.

RESULTS AND DISCUSSION

Characteristics of MIMs

FTIR spectra of the MIMs sample (Figure 1) were inspected to verify the successful preparation of MIMs. A broad absorption band at 3550 and 1635 cm^{-1} is corresponding to the stretching vibration of O—H bonds. The observed features at 2900, 1730, and 1160 cm^{-1} indicate =C—H, —C=O, and —C—O— stretching vibrations, respectively. The distorting vibrations of =C—H are reflected around 1450–1280 cm^{-1}. These results suggested that the functional monomer (MAA) and cross-linker (EGDMA) successfully polymerized, yielding MIMs.

In the preparation of MIMs, the optimum molar ratios of template/monomer/cross-linker were 1:8:40 and the optimum porogen was toluene. SEM image of MIMs shows that spherical particles with fairly similar diameters ranging from 1 to 2 μm were obtained in the optimum condition.

Loading Step of MISPE

Commonly, synthesis solvent of MIMs was used as loading solvent in MISPE, because the synthesis solvent provided the most adaptive environment for MIMs to adsorb analytes. However, our objective was to use the MIMs to extract aconitine from body fluid samples, and a water-based loading solvent was required. Solvents of methanol : water (9:1, 8:2, 7:3, 6:4, 5:5, v/v) as loading solvents were investigated in order to optimize adsorption condition for body fluid samples. The results showed that aconitine did not bled at all in these loading solutions. Considering that we would add methanol into commensurate volume of serum sample for protein precipitation, the solvent of methanol : water (5:5, v/v) was chosen as loading solvent for subsequent experiments.

Adsorption Capability

Totally, 2 μg mL^{-1} of Aconitine solution was continuously loaded into MIMs and NIMs cartridges 1 mL followed by 1 mL until aconitine was not adsorbed any more. Figure 3 indicated that 100% of aconitine in the first 25 mL of loading solution

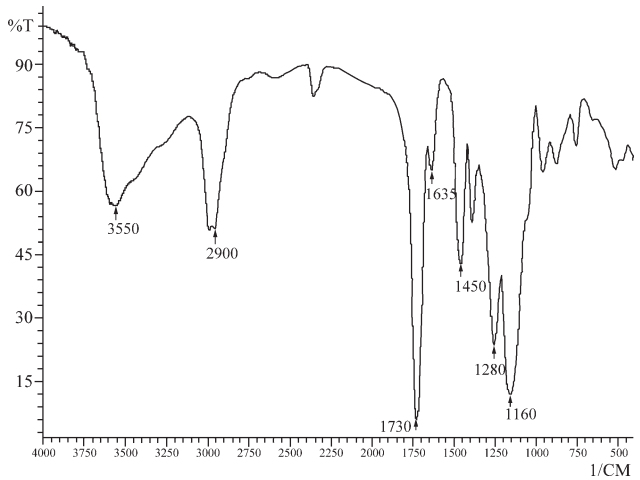

Figure 1. FTIR spectra of MIMs.

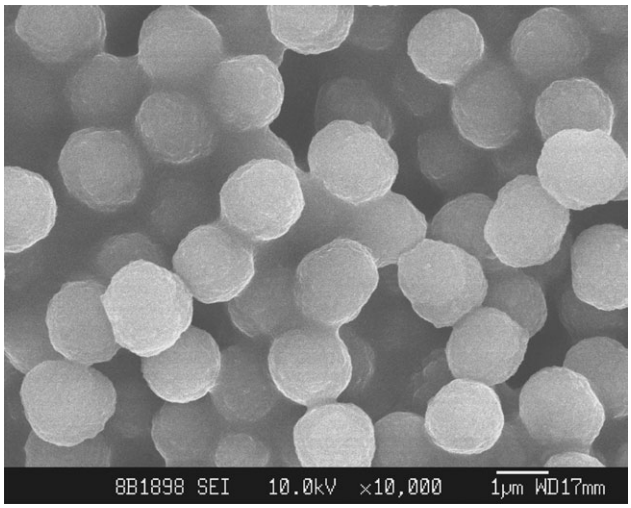

Figure 2. SEM image of MIMs (magnification : ×10,000).

was adsorbed on the MIMs cartridge, while the 100% of aconitine only in the first 5 mL of loading solution was adsorbed on the NIMs cartridge. After 57 mL being loaded, the MIMs cartridge reached saturated, and the adsorption amount was 0.802 mg g^{-1}, while the adsorption amount of the NIMs cartridge was 0.466 mg g^{-1}. When the loading volume was below 10 mL and the amount of aconitine in the loading solution was very low, the MIMs cartridge still did not reach saturated while the NIMs gradually reached saturated because of its nonspecific absorbability. As the MIMs were prepared for using aconitine as template molecules, the adsorption of MIMs for aconitine was owed chiefly to specific adsorption, including a small quantity of nonspecific adsorption. But the adsorption of NIMs for aconitine was all nonspecific adsorption.

Washing Step of MISPE

To eliminate impurities, after loading with 1 mL of aconitine solution (2 μg mL^{-1}), cartridges were washed with a series of different ratios of methanol-water solvent, and the results

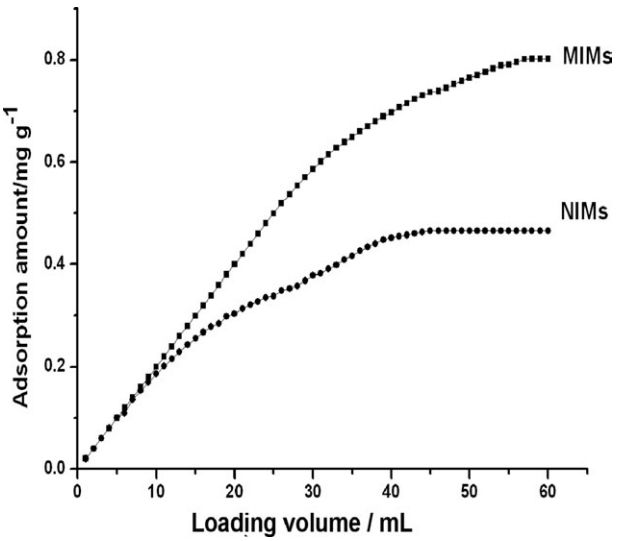

Figure 3. Adsorption curves of aconitine on MIMs and NIMs cartridges.

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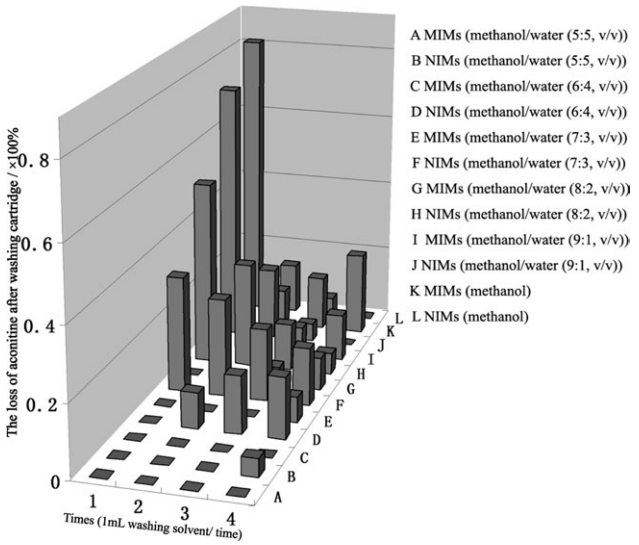

Figure 4. Effect of different washing solvents on eliminating impurities in MIMs cartridge and NIMs cartridge.

(Figure 4) indicated that more aconitine was washed out with the increase of the proportion of methanol which may weaken the hydrophobic interactions between the adsorbent and aconitine. As the adsorption of NIMs for aconitine was all nonspecific adsorption, aconitine was lost obviously even the NIMs were washed with solvent of methanol : water (6:4, v/v). When the NIMs were washed with solvent of methanol : water (8:2), aconitine was little retained; however, 83% of aconitine was retained on MIMs cartridge which was also washed with solvent of methanol : water (8:2). And 78% of aconitine was still retained on MIMs cartridge when MIMs were washed with solvent of methanol : water (9:1). The results also demonstrated the interactions between aconitine and MIMS were much stronger than that between aconitine and NIMS. After that the MIMs and NIMs were washed with 3 mL of methanol : water (7:3, v/v), only 21% of aconitine was retained on the NIMs cartridge, while 100% of aconitine was retained on the MIMs cartridge, yielding an imprinting factor (IF) of 4.76. For the 4thmL, the loss of aconitine on the MIMs cartridge was 7% (>5%). Solvent of methanol : water (7:3, v/v) could disrupt the nonspecific interactions between aconitine and the NIMs as well as

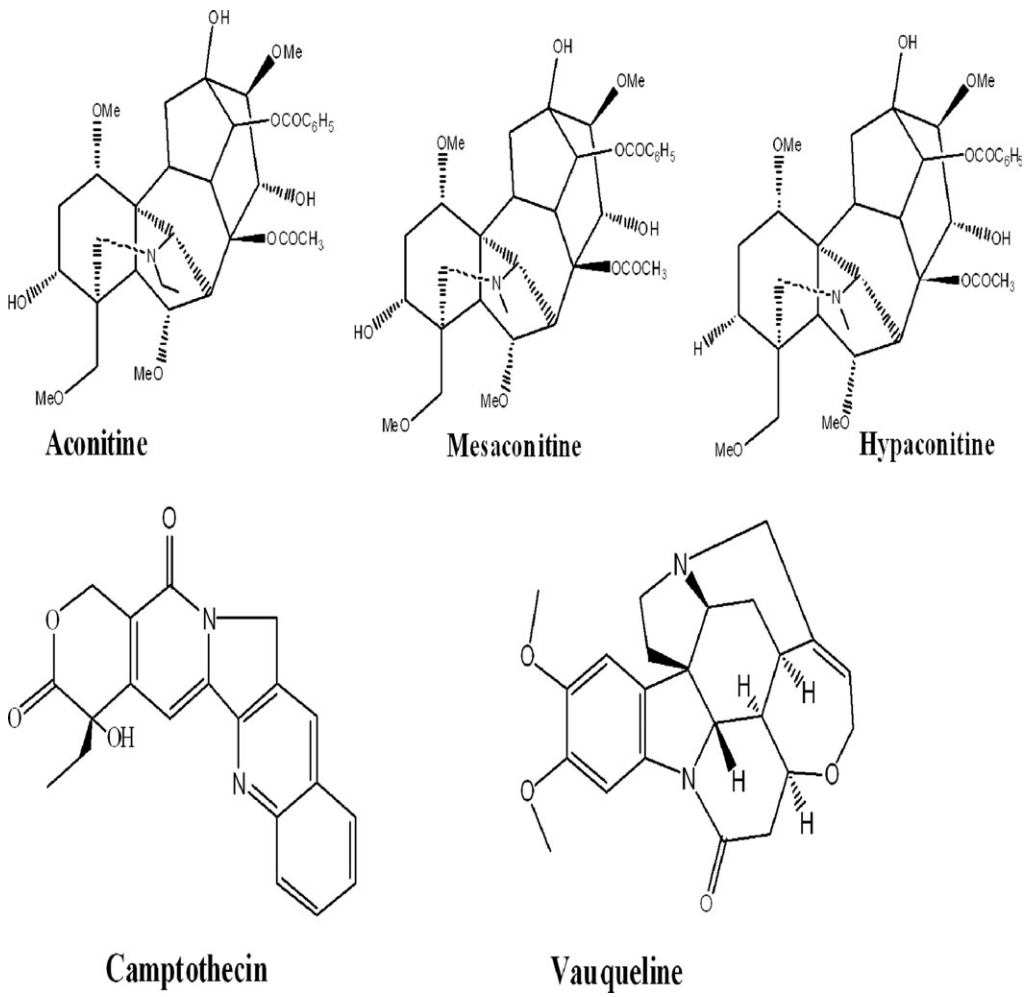

Figure 5. Structures of aconitine, mesaconitine, hypaconitine, camptothecin, and vauqueline.

Table I. Comparison of Recoveries of Five Analytes after MIPSE

	Aconitine	Mesaconitine	Hypaconitine	Camptothecin	Vauqueline
Loading/%	0	0	0	28.1	6.5
Washing/%	0	0	0	56.5	19.3
Eluting/%	98.7	99.5	95.9	12.4	73.5
Recovery/%	98.7	99.5	95.9	97.0	99.3

The MISPE conditions: methanol were: loading solvent, methanol : water (5:5, v/v); washing solvent, methanol : water (7:3, v/v); eluent, methanol : acetic acid (7:3, v/v).

retain the specific interactions between aconitine and the recognition sites in the MIMs. Methanol may be able to remove the water-insoluble interfering agents. Thereby, we chose 3 mL of methanol : water (7:3, v/v) as washing solvent in further experiments.

Eluting Step of MISPE

Different ratios of Methanol : acetic acid was investigated as eluent because acetic acid could elute some strongly bound analytes.[37–39] To elute aconitine from SPE cartridges thoroughly, 3 mL of the eluting solvent of methanol : acetic acid (9:1, v/v) or 2 mL of methanol : acetic acid (8:2, v/v) could elute aconitine completely, while 1 mL of methanol : acetic acid (7:3, v/v) also eluted aconitine out. As the C18 column must be used in the pH range of 2–10, in order to protect HPLC column, we did not add acetic acid anymore; and methanol : acetic acid (7:3, v/v) was selected as eluent for further experiments.

MISPE Selectivity

Three aconitum alkaloids and two nonaconitum poisonous alkaloids (Figure 5) were chosen to evaluate the selectivity of MIMs.

The mixed solution of the three aconitum alkaloids or mixture of the two nonaconitum poisonous alkaloids was loaded into the MIMs cartridge and selectivity of the MIMs was investigated. The results were showed in Table I.

The results showed that in the loading step the recoveries of camptothecin and vauqueline were 28.1, 6.5%, respectively. And in washing step the recoveries of camptothecin and vauqueline were 56.5, 19.3%, respectively. However the recoveries of aconitine, mesaconitine and hypaconitine were none in loading and washing steps. Mesaconitine and hypaconitine are aconitum alkaloids and are most structurally similar to aconitine. Although aconitine was used as template, they were all high-recovered by MISPE. However, the two kinds of nonaconitum poisonous alkaloids are less structurally similar to aconitine and bled in the loading and washing steps. Vauqueline lost less than camptothecin because the stereostructure of vauqueline was much more like aconitine, compared with camptothecin.

As MIPs were synthesized in the presence of aconitine and after removal of aconitine, maybe there were resultant cavities, which complement aconitine in size, shape, and arrangement of functional groups (from MAA) and allowed MIPs to rebind aconitine molecules. This indicated the MIPs had high affinity to aconitum alkaloids which matched the binding cavities in MIPs.

Table II. Recoveries of Aconitine, Hypaconitine, and Mesaconitin in Serum Samples

	Recovery/%		
	Aconitine	Hypaconitine	Mesaconitine
2.0 μg mL^{-1}	92.9 ± 2.6	91.2 ± 4.8	92.3 ± 0.3
1.0 μg mL^{-1}	94.2 ± 7.8	94.5 ± 4.0	90.7 ± 1.2
0.1 μg mL^{-1}	89.0 ± 5.9	89.1 ± 3.2	89.7 ± 6.2

HPLC conditions: mobile phase was a mixture of methanol : water : ammonium acetate : acetic acid (545:450:1.2:5.0, v/v/v/v); flow-rate: 0.5 mL/min.

Serum Samples

The investigation of working curves were performed by analyzing spiked serum samples with the concentration range from 2.0 to 0.1 μg mL^{-1}. Their recoveries (>89%) were showed in Table II. Working curves were constructed by plotting the peak area (Y) of the aconitum alkaloid against its concentration (X). The correlation coefficients of the curves for aconitine, hypaconitine, and mesaconitine were 0.9958, 0.9935, and 0.9984, respectively. The sample generated with MISPE showed a particularly well-defined chromatographic trace (Figure 6).

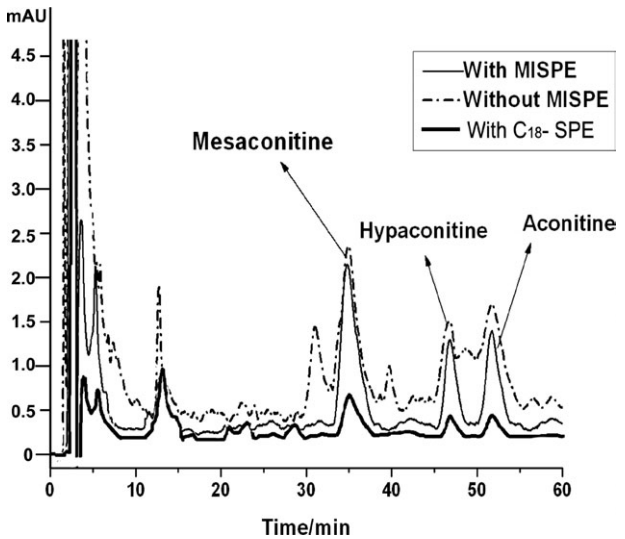

Figure 6. HPLC trace of serum samples on C18 column. HPLC conditions: mobile phase was a mixture of methanol : water : ammonium acetate : acetic acid (545:450:1.2:5.0, v/v/v/v); flow-rate: 0.5 mL/min; injected volumn : 10 μL.

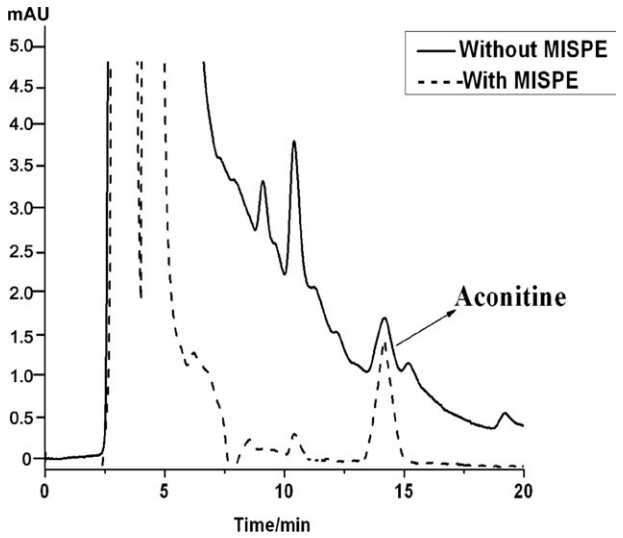

Figure 7. HPLC trace of urine samples on C18 column. HPLC conditions: mobile phase was a mixture of methanol : water : ammonium acetate : acetic acid (545:450:1.2:5.0, v/v/v/v); flow-rate: 0.5 mL/min; injected volumn : 10 μL.

The limits of detection (LODs) defined as signal-to-noise ratio (S/N) of 3:1, are 16.7, 18.3, 10.2 μg mL^{-1} for aconitine, hypaconitine, and mesaconitine in serum samples, respectively.

Urine Samples

After being filtrated, the spiked urine sample (2 μg mL^{-1}) was intended to be dealt with MISPE. Some organic solvent (like acetic acid or acetone) was required in washing solvent, since the interference of human urine sample was rather serious. Mixtures of methanol–acetone–water (70:1:29, v/v/v), methanol–acetone–water (70:3:27, v/v/v) and methanol–acetone–water (70:5:25, v/v/v) were investigated as washing solvents. The results indicated that methanol–acetone–water (70:1:29, v/v/v) failed to purify the urine sample. A low recovery of 80.5% of aconitine was obtained with methanol–acetone–water (70:5:25, v/v/v) as washing solvent. However, methanol–acetone–water (70:3:27, v/v/v) could wash impurities out and a relatively high recovery of 86.4% was gained, so it was chosen as washing solvent of urine samples, while MISPE process was with methanol–acetone–water (70:3:27, v/v/v) as washing solvent, a baseline separated HPLC trace (Figure 7) was obtained.

CONCLUSIONS

In this study, we presented a system protocol of preparing and evaluating aconitine imprinted polymer microspheres and its application to purify human serum and urine samples. The imprinted microspheres synthesized by precipitation polymerization showed high selectivity among different kinds of poisonous alkaloids. Moreover, the MIMs extracted the three aconitum alkaloids with high recoveries from human serum samples. The MIMs separated aconitine alkaloids from human urine samples with a well-defined chromatographic trace after optimization of the MISPE procedures. The MISPE could be applied in field of clinical detection.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (No. 20875034) and the Science and Technology Plan Projects of Guangdong Province (No. 2008B030303073).

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